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Original Article

Stimulatory effect of cytochalasin D on antigen-induced phospholipase D activation in a murine mast cell model (RBL-2H3)

Yoshikatsu Nakamura,[1] Shigeru Nakashima,[2] Katsuhiro Ojio,[1] Yatsuji Ito,[1] Kazuki Hayakawa,[1] Hideo Miyata[1] and Yoshinori Nozawa[2]

[1]Department of Otolaryngology, [2]Department of Biochemistry, Gifu University School of Medicine, Gifu, Japan

Abstract

To investigate the possible involvement of cytoskeletal components in antigen (Ag)-mediated activation of phospholipase D (PLD) in rat basophilic leukemia (RBL-2H3) cells, the effects of cytochalasin D, which is known to interfere with actin organization in various cells, on Ag-induced PLD activity were examined. Cytochalasin D, at concentrations that induced distinct shape changes of RBL-2H3 cells, enhanced the Ag-induced 5-HT (serotonin) release and formation of phosphatidylbutanol (PBut), a specific and stable metabolite produced by PLD activity in a concentration-dependent manner. Concomitantly, Ag-induced 1,2-diacylglycerol (DG) accumulation as well as phosphatidic acid (PA) production were increased by the drug. In contrast, cytochalasin D had no effect on PLD activation in response to phorbolmyristate acetate, an activator of protein kinase C (PKC), and Ca^{2+} ionophore A23187. These results suggest that cytoskeletal components may modulate Ag-induced PLD activation upstream of PKC and Ca^{2+} in RBL-2H3 cells.

Key words: cytochalasin intracellular signal transduction, mast cells, phospholipase D.

Correspondence: Dr Yoshikatsu Nakamura, Department of Otolaryngology, Gifu University School of Medicine, Tsukasamachi-40, Gifu, 500-8705, Japan.
Email: <yosikatu@gumail.cc.gifu-u.ac.jp>

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Introduction

Mast cells and basophils express the type I cell surface receptor for Fc domains of class E immunoglobulin (IgE). Upon antigen (Ag) stimulation, cross-linking of receptor–IgE complexes induces release of chemical mediators such as histamine, serotonin, leukotrienes and prostaglandins in mast cells. Rat basophilic leukemia (RBL-2H3) cells share many of the properties of mucosal mast cells and are widely used as a model for the study of high-affinity IgE Fc receptor (FcεRI)-mediated signaling events. Aggregation of FcεRI leads to activation of signal transducing phospholipases.[1,2] Hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) produces phosphatidic acid (PA), which can be further converted to 1,2-diacylglycerol (DG).[3] The receptor-mediated PLD activation is thought to be involved in a variety of cellular responses: rapid response, such as secretion and superoxide generation, as well as long-term responses, such as proliferation, differentiation and apoptosis.[3–8] Antigen-stimulated PLD activation has been shown to contribute to the sustained DG formation that may induce a long-lasting activation of protein kinase C (PKC).[9,10] Possible involvement of Ca^{2+}, PKC, protein tyrosine kinase (PTK), and ADP ribosylation factor (Arf) in Ag-mediated PLD activation has been demonstrated.[11–15] Recently, Rho family GTP-binding proteins, Rho, Rac and Cdc42, which are thought to be involved in the formation of actin stress fiber, lamellipodia and filopodia, have also been regarded as regulators of PLD activity.[16–18] In contrast, fodrin, a non-erythroid form of spectrin, is reported as an inhibitor of PLD activity.[19] In neutrophils, cytochalasin B is necessary

for maximal activation of PLD by chemo-attractants such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLP).[20,21] These results suggest the possible involvement of cytoskeletal components in the regulation of PLD activity.

Cytochalasins, a group of fungal metabolites, permeate cell membranes and cause cells to stop ruffling and to round up.[22,23] They appear to bind to the barbed end of the actin filament and to inhibit both the association and the dissociation of actin molecules at that end. The stoichiometry of binding is about one cytochalasin per actin filament.[24] In addition to binding actin, cytochalasins A and B also inhibit monosaccharide transport across the plasma membrane.[25] However, cytochalasins C, D, E, and H do not affect sugar transport.

In the present study, to gain further insight into the mechanism for the Ag-stimulated PLD activation, involvement of cytoskeleton was examined by the use of cytochalasin D in RBL cells. The results obtained suggest that the inhibition of assembly of actin filament increases Ag-stimulated PLD activation in RBL cells.

METHODS

Materials

Monoclonal mouse anti-dinitrophenyl (DNP) IgE was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). *Ascaris suum* Ag coupled with 2,4-dinitrophenyl (DNP-As) was synthesized as described previously.[2] Eagle's minimum essential medium (MEM), penicillin and streptomycin were obtained from Gibco Laboratories (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, CA, USA). [^{14}C]-5-Hydroxytryptamine binoxalate (serotonin, 5-HT) (1.85 MBq/mmol) and Aquasol-2 were purchased from Dupont-New England Nuclear (Boston, MA, USA). [9,10-^{3}H]-Palmitic acid (1.94 TBq/mmol) were from Amersham International (Buckinghamshire, UK). 4β-Phorbol 12-myristate 13-acetate (PMA) was from Sigma (St Louis, MO, USA). A23187 was from Calbiochem (San Diego, CA, USA). Silica gel LK6D plates were obtained from Whatmann (Clifton, NJ, USA). Other reagents were of analytical grade. Phosphatidylbutanol (PBut) standard was synthesized from egg PC using crude cabbage PLD according to the method of Yang *et al*.[26]

Cell culture

Monolayer culture of RBL-2H3 cells was maintained in MEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 95% air/5% CO_2 at 37°C.[1]

Serotonin release

RBL-2H3 cells were incubated with [^{14}C]-5-HT (370 Bq/1.5×10^5 cells) for 16 h at 37°C. For sensitization, anti-DNP IgE (0.3 μg/mL) was incubated for the last 4 h of radiolabeling. The cultures were washed twice and equilibrated in Tyrode-HEPES solution (NaCl 134 mmol/L, $NaHCO_3$ 12 mmol/L, KCl 2.9 mmol/L, $MgCl_2$ 1 mmol/L, $CaCl_2$ 1.8 mmol/L, NaH_2PO_4 0.36 mmol/L, glucose 5.6 mmol/L, HEPES 10 mmol/L, 0.1% bovine serum albumin (BSA), pH 7.40) for 10 min at 37°C prior to pretreatment of cytochalasin D. After pre-incubation for 10 min with various concentrations of cytochalasin D, the cells were stimulated with Ag (100 ng/mL). Incubation was stopped promptly by removing the medium, followed by addition of 0.2 mL of ice-cold phosphate-buffered saline (PBS) with 1% Triton X-100. The radioactivity in the medium and the cells (Triton X-100 extract) was determined by a liquid scintillation counter (Beckmann LS 6500, Fullerton, CA, USA) with 5 mL Aquasol-2. Serotonin release was determined as the percentage of radioactivity recovered in medium to the total radioactivity.

Analysis of lipid metabolism

Phospholipase D activity in response to Ag was determined by measuring the formation of [^{3}H]-PBut in the presence of butanol (0.3%, v/v) as described previously.[27] RBL-2H3 cells (1.5×10^6 cells/35 mm dishes) were labeled with [^{3}H]-palmitic acid (74 kBq/dish) for 20 h. After removal of the medium, the dishes were rinsed three times and equilibrated in Tyrode-HEPES solution. For measurement of PLD activity, cells were incubated in the presence of 0.3% butanol. However, in order to examine the production of PA and 1,2-DG, butanol was not included in the incubation medium. Incubations were stopped by removing medium, followed by the immediate addition of 1 mL ice-cold PBS/methanol (2 : 5, v/v) mixture to the culture dishes. Cells were scraped from the dishes with a rubber policeman, with an additional 1 mL PBS/methanol mixture, and transferred to a tube containing 700 μL chloroform. The lipid extraction was performed according to the method of Bligh and Dyer.[28] [^{3}H]-Phosphatidylbutanol and [^{3}H]-PA were separated by one-dimensional thin-layer chromatography with silica gel LK6D plates in a solvent system of upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water

(13 : 2 : 3 : 10, v/v).[29] [^{3}H]-Diacylglycerol was separated in a solvent system of chloroform/acetone (96 : 4, v/v). The area corresponding to these lipids, identified by comigration with lipid standards, was scrapped off the plate and the radioactivity was determined in a liquid scintillation counter (Beckmann LS 6500).

Data analysis

Data in the figures are expressed as means ± SD of two experiments, each performed in duplicate. To determine the levels of significance between sets of data, Bonferroni method was employed using StatView version 4.5 (Abcans Concepts Inc.).

Results

Effects of cytochalasin D on cell morphology and serotonin release

It has been reported that cytochalasin D caused depolymerization of actin cytoskeleton.[30] To delineate the implication of cytochalasin D in the Ag-mediated signaling processes in RBL-2H3 cells, we first examined its effect on cell morphology. As expected, cytochalasin D induced morphological changes. Cell shape changes were initiated 1 h after addition of cytochalasin D (2 μmol/L), followed by distinct rounding out at 4 h (Fig. 1b).

The secretory response was measured in [^{14}C]-5-HT-labeled RBL-2H3 cells. Cytochalasin D (2 μmol/L) had no effect on spontaneous [^{14}C]-5-HT release. When RBL-2H3 cells were stimulated with 100 ng/mL Ascris Ag for 30 min, nearly 54% of [^{14}C]-5-HT was released. Cytochalasin D (0.2–2 μmol/L) enhanced Ag-induced [^{14}C]-5-HT release in a concentration-dependent manner and the maximal stimulatory effect was obtained at 1 μmol/L, where nearly 85% of [^{14}C]-5-HT release was observed (Fig. 2).

Effect of cytochalasin D on Ag-induced PLD activation

Phospholipase D hydrolyzes phospholipids, especially PC to PA and choline. In addition to hydrolysis, PLD also catalyzes specific transphosphatidylation reaction in which a primary alcohol acts as an acceptor in place of H_2O.[7] The production of phosphatidylalcohol is often used as an indicator for the activity of PLD. The formation of PBut in the presence of butanol was measured to monitor Ag-stimulated PLD activity in RBL-2H3 cells. When [^{3}H]-palmitic acid-labeled RBL-2H3 cells were stimulated with Ag (100 ng/mL) in the presence of 0.3% butanol, [^{3}H]-PBut was rapidly produced within the first 2 min and reached a plateau at 10 min.[11] In subsequent experiments, [^{3}H]-PBut formation was measured at 10 min after Ag stimulation. [^{3}H]-Phosphatidylbutanol increased from an unstimulated control level of 0.12% to 0.23% (nearly two-fold increase) at 10 min after Ag addition (Fig. 3). When cells were incubated with various concentrations (0.2–2 μmol/L) of cytochalasin D for 10 min, Ag-induced [^{3}H]-PBut formation was augmented in a concentration-dependent manner (Fig. 3). The maximal stimulatory effect was obtained at 1 μmol/L cytochalasin D. However, cytochalasin D had no effect on the basal [^{3}H]-PBut level.

Effects of cytochalasin D on Ag-induced DG and PA productions

As reported in some types of cells,[1,2] Ag induced a biphasic DG production in RBL-2H3 cells.[10,11] The second

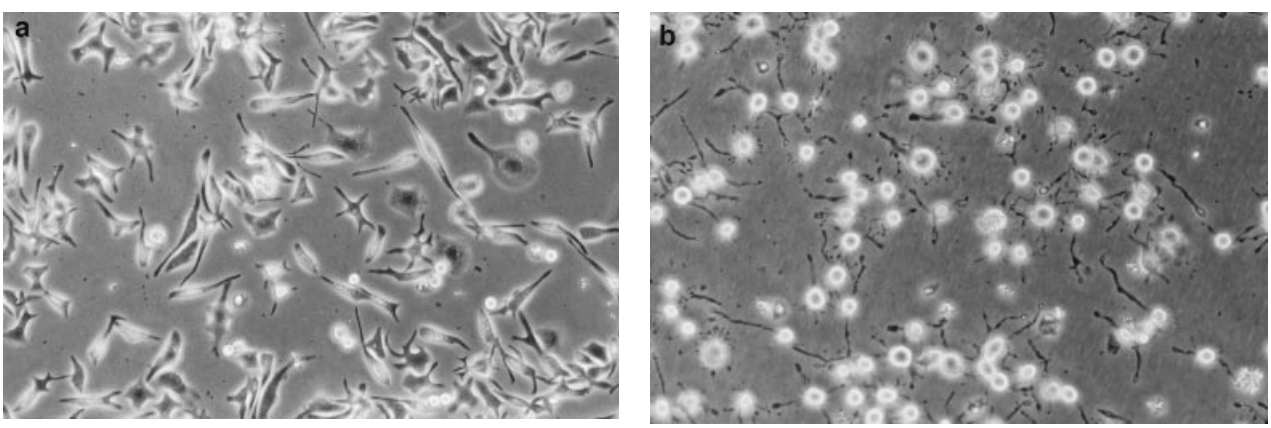

Fig. 1 Morphological changes of RBL-2H3 cells treated with cytochalasin D. Cells were incubated without (a) or with (b) 2 μmol/L cytochalasin D for 4 h. The photos shown are representative of two separate experiments.

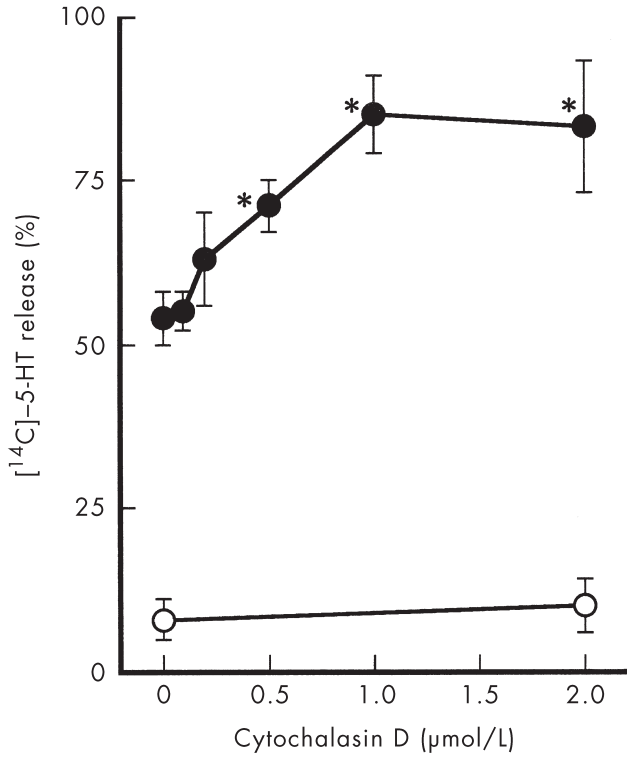

Fig. 2 Effects of cytochalasin D on antigen (Ag)-induced serotonin (5-HT) release. RBL-2H3 cells were labeled with [^{14}C]-5-HT for 16 h. After removing unincorporated radiolabels, cells were incubated with the indicated concentrations of cytochalasin D for 10 min before stimulation. The cells were stimulated with 100 ng/mL Ag for 30 min (●). Release of [^{14}C]-5-HT was calculated as described in Methods. (○), control. Data are the mean ± SD from two experiments, each performed in duplicate. Significant differences between the presence and the absence of cytochalasin D were statistically analyzed by the Bonferroni method. *$P < 0.05$.

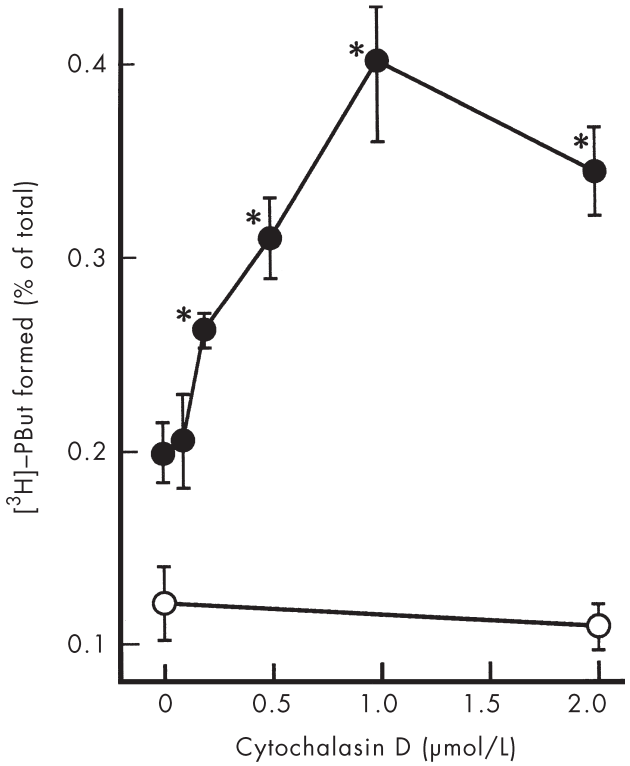

Fig. 3 Effects of cytochalasin D on antigen (Ag)-induced phosphatidylbutanol (PBut) formation. RBL-2H3 cells were labeled with [^{3}H]-palmitic acid for 20 h. After removal of unincorporated radiolabels, cells were incubated with the indicated concentrations of cytochalasin D for 10 min before stimulation. The radiolabeled cells were then stimulated with 100 ng/mL Ag in the presence of 0.3% butanol for 10 min (●). (○), control. After lipid extraction, PBut was separated on thin-layer chromatography plates. Details are described in Methods. Data are the mean ± SD from two experiments, each performed in duplicate. Significant differences between the presence and the absence of cytochalasin D were statistically analyzed by the Bonferroni method. *$P < 0.05$.

large sustained phase is derived mainly from PC breakdown. To further investigate the effects of cytochalasin D on Ag-induced phospholipid turnover, production of both DG and PA was examined. [^{3}H]-Palmitic acid-labeled RBL-2H3 cells were stimulated with Ag (100 ng/mL) in the absence of 0.3% butanol, and [^{3}H]-DG and [^{3}H]-PA were rapidly produced within the first 2 min, reaching a peak at 5 min, as previously described.[31,32] When cells were incubated with cytochalasin D (1 μmol/L) for 10 min prior to stimulation, Ag-induced [^{3}H]-DG accumulation (Fig. 4a), as well as [^{3}H]-PA production (Fig. 4b), was increased. Cytochalasin D at 1 μmol/L enhanced the production of both DG and PA by about two-fold.

Effect of cytochalasin D on PMA- or A23187-induced PLD activation

We further examined whether cytochalasin D exerted stimulatory effects on the receptor-bypass stimuli such as PMA and Ca^{2+} ionophore. 4β-Phorbol 12-myristate 13-acetate, a well-known activator of PKC, and Ca^{2+} ionophore A232187 caused PLD activation in RBL-2H3 cells as previously observed.[11] Phosphatidylbutanol formation increased from 0.10% (basal unstimulated level) to 0.42% with PMA (100 nmol/L) and 0.70% with A23187 (1 μmol/L). However, cytochalasin D (0.1 or 1 μmol/L) did not affect PMA- and A23187-induced [^{3}H]-PBut formation (Fig. 5).

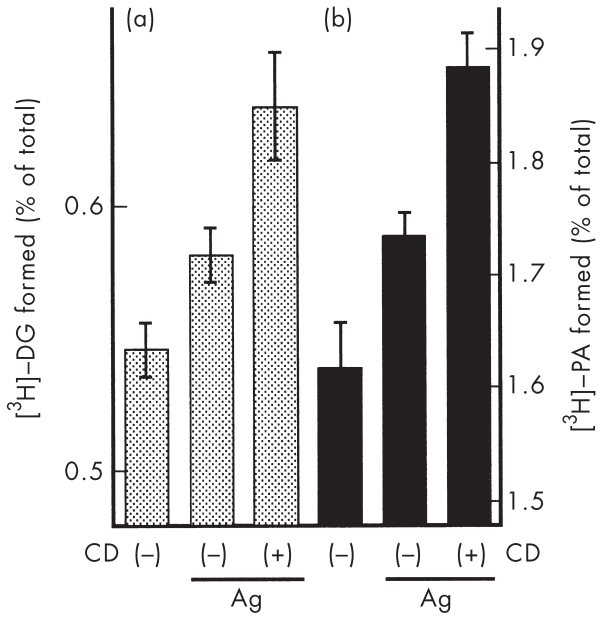

Fig. 4 Effects of cytochalasin D on antigen (Ag)-induced formation of 1,2-diacylglycerol (DG) (a) and phosphatidic acid (PA) (b). RBL-2H3 cells were labeled with [^{3}H]-palmitic acid for 20 h. After removal of unincorporated radiolabels, cells were incubated with the indicated concentrations of cytochalasin D for 10 min before stimulation. The radiolabeled cells were then stimulated with 100 ng/mL Ag for 5 min. After lipid extraction, 1,2-DG and PA were separated on thin-layer chromatography plates. Details are described in Methods. Data are the mean ± SD from two experiments, each performed in duplicate.

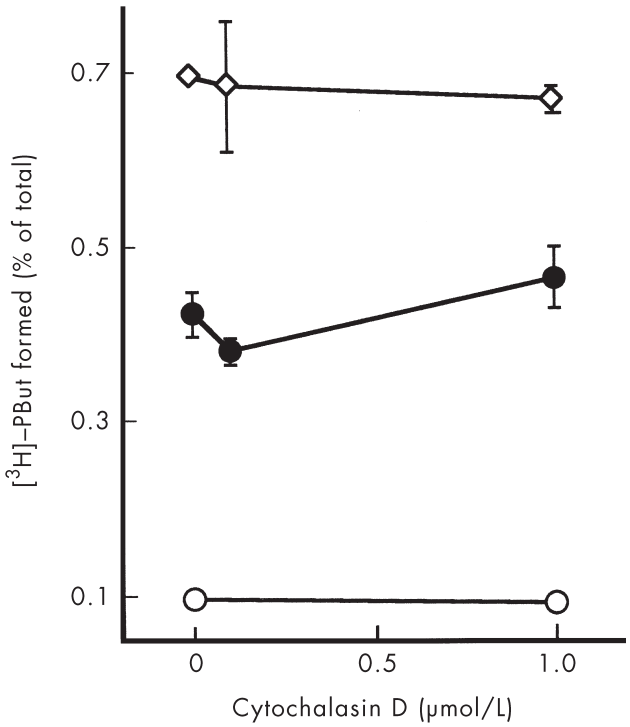

Fig. 5 Effects of cytochalasin D on phosphatidylbutanol (PBut) formation induced by 4β-phorbol 12-myristate 13-acetate (PMA; ●) or A23187 (◇). RBL-2H3 cells were labeled with [^{3}H]-palmitic acid for 20 h. After removal of unincorporated radiolabels, cells were incubated with the indicated concentrations of cytochalasin D for 10 min before stimulation. The radiolabeled cells were then stimulated with 100 nmol/L PMA or 1 μmol/L A23187 in the presence of 0.3% butanol for 10 min. Details are described in Methods. (○), control. Data are the mean ± SD from two experiments, each performed in duplicate.

Discussion

The receptor-mediated PLD activation is thought to be involved in a variety of cellular responses such as secretion and superoxide generation.[3–8] Recent cDNA cloning studies have revealed the existence of at least two isozymes (PLD1 and PLD2) in mammalian cells.[33–37] However, details of their activation mechanisms in response to external stimuli are not fully disclosed. Possible involvement of Ca^{2+}, PKC, or protein tyrosine kinase in Ag-induced PLD activation has been demonstrated.[13,14,27] Moreover, Rho family GTP-binding proteins, Rho, Rac and Cdc42, which are involved in the formation of actin stress fibers, take part in PLD activation.[16–18] Our previous study demonstrated that *Clostridium difficile* Toxin B, which blocks the function of Rho family GTP-binding proteins, inhibited Ag-mediated PLD activation in RBL-2H3 cells.[18] In contrast, fodrin, a non-erythroid form of spectrin, was reported as an inhibitor of PLD activity.[20] These results suggest the possible involvement of cytoskeletal components in the regulation of PLD activity. In order to clarify the role of actin cytoskeleton, the effects of cytochalasin D, which interferes with actin organization, on Ag-induced PLD activity have been examined. The pretreatment of RBL-2H3 cells with cytochalasin D resulted in enhancement of Ag-mediated PLD activation. Moreover, the production of 1,2-DG as well as PA was also increased by cytochalasin D. These results suggest that actin cytoskeleton may negatively regulate Ag-induced PLD activation.

It is well known that Ca^{2+} mobilization plays a crucial role in the regulation of secretory response and PLD activation in RBL-2H3 cells.[10–14] However, it has been reported that cytochalasin D had no effect on Ag-induced Ca^{2+} mobilization.[38] Several recent studies have indicated that phosphatidylinositol 4,5-bisphosphate (PIP_2) is

an essential cofactor for PLD activity in *in vitro* assay systems.[39,40] It has been reported that RhoA, which is listed as one of the activators for PLD, activates phosphatidylinositol-4-phosphate 5-kinase, which synthesizes PIP_2.[41] The various actin-binding proteins such as profilin or α-actinin are known to bind PIP_2.[42,43] Therefore, it may be possible that cytochalasin D changed the level of free PIP_2 in the cells through modulation of the interaction between actin and actin-binding proteins. However, cytochalasin D had no effect on 100 nmol/L PMA- or 1 μmol/L A23187-induced PLD activation. Phosphatidylinositol 4,5-bisphosphate is a prerequisite for PLD activation by PKC in *in vitro* assay systems. Therefore, the stimulatory effect of cytochalasin D on Ag-induced PLD activation may occur to be dependent of the change of PIP_2 level. Although details of the inhibitory mechanisms are subjects for future study, the present results suggest that cytoskeletal components modulate Ag-mediated PLD activity upstream of PKC and Ca^{2+} in RBL-2H3 cells.

Since primary alcohols, ethanol, butanol etc. that inhibit the production of PA by PLD, inhibit Ag-mediated secretory responses,[13] PLD activity is thought to be closely coupled with secretory response. Similar findings are reported in various types of cells.[7,21] Activation of PLD1 by Arf, which is known as a component of vesicular trafficking from endoplasmic reticulum (ER) to Golgi apparatus, supports this notion.[44,45] Moreover, recent studies have revealed that PLD1 localizes in ER and/or Golgi apparatus.[34,46,47] In the cells treated with cytochalasin D, there was a positive correlation between increase in PLD activity and 5-HT release. Therefore, the present results provide further evidence showing the involvement of PLD in secretory responses.

In summary, cytochalasin D, which blocked actin assembly, increased Ag-induced PLD activity and 5-HT release in RBL-2H3 cells. These results suggest the possible involvement of actin cytoskeleton in the regulation of PLD activity.

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